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Nucleic Acid Detection as a Diagnostic Tool in Polyomavirus JC Induced Progressive Multifocal Leukoencephalopathy

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Procedures involved in the diagnosis of JC virus central nervous system infection range from detection of virus specific products in biopsy material to demonstration of viral DNA in cerebrospinal fluid by PCR. Despite the fact that PCR is the most sensitive method for the detection of virus in clinical specimens, diagnostic evaluation is increasingly difficult in view of the possible subclinical activation of persistent JCV infection in the central nervous system of high risk patients. Therefore, PML diagnosis by molecular detection of JCV DNA in biopsy material was compared with diagnosis by PCR on CSF of patients with and without PML. Evaluation of the diagnostic techniques revealed that stereotactic biopsy based PCR diagnosis at present combines speed and sensitivity with the highest specificity available. Although the non invasive technique of JCV detection in CSF by PCR is even more sensitive leading to detection of about 20 genome equivalents per 1 µl of CSF, the specificity of the method is limited by subclinical presence of JCV DNA in CSF of neurologically asymptomatic HIV infected patients. Additionally, autopsy proven PML cases remaining JCV negative in PCR on CSF become a common finding. Therefore, in cases where biopsy is not performed, diagnosis of PML can only be achieved in combination with neurological and radiological diagnosis. ***J. Med. Virol. 54:196–203, 1998.***

KEY WORDS: PML; CNS disease; JCV persistence

INTRODUCTION

Infection with the human polyomavirus JC (JCV) leads to lifelong asymptomatic persistence, occasionally causing progressive multifocal leukoencephalopathy (PML) in severely immunosuppressed patients [Dörries, 1997]. The disease is characterized by disseminated development of demyelinated plaques in the white matter and adjacent areas. At the rim of these foci virus accumulates to high concentrations followed by cytolytic destruction of oligodendrocytes [Weber et al., 1994]. Besides lymphoproliferative diseases [Brooks and Walker, 1984], AIDS is the major underlying disorder in PML with a steadily increasing incidence since 1982. With 5% to 20% of AIDS patients succumbing eventually to the disease [Moret et al., 1993] AIDS-associated PML is the third most common process producing focal CNS lesions in these patients [Ciricillo and Rosenblum, 1990]. Although a number of different approaches to treatment of PML is discussed, in contrast to other opportunistic CNS disorders the outcome is fatal in almost all cases.

The diagnosis of PML on the basis of neurological evaluation and neuroimaging of the brain is followed by definitive laboratory diagnosis on biopsy material with various techniques [Kepes et al., 1975; Gibson et al., 1986; Aksamit et al., 1987; Gosztonyi and Cervos-Navarro, 1988; Karahalios et al., 1992; Ferrante et al., 1995]. Although this approach is widely used, the technical equipment is costly, and the methods are laborious and time consuming. Additionally, they are limited by the problems associated with the use of invasive techniques in late stages of AIDS or in the uncharacteristic relapsing course of disease. With the need for differentiation from other opportunistic CNS infections in AIDS patients, it becomes essential to introduce

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methods that allow an early and fast routine diagnosis. Therefore, several laboratories aimed for the development of less invasive diagnostic methods. The well known highly efficient multiplication of JCV in the CNS in the course of PML led to the suggestion that disseminated localization of active plaques might be associated with shedding of virus particles into the cerebrospinal fluid (CSF). Assuming virus can be regularly detected in CSF, several laboratories confirmed the presence of JC virus particles or specific DNA segments in the CSF of PML patients by electron microscopy [Orefice et al., 1993] or molecularly by PCR [Henson et al., 1991; Moret et al., 1993; Weber et al., 1994; DeLuca et al., 1996].

Although PCR is the most sensitive method for the detection of virus in tissue and body fluids to date, the diagnostic evaluation of the results becomes increasingly difficult in view of activation states of persistent JCV infections in non-PML patients [Elsner and Dörries, 1992; Quinlivan et al., 1992; White et al., 1992; Ferrante et al., 1995]. Since subclinically shed virus might produce false positive diagnosis of PML during immunosuppressive states we compared results of molecular detection methods for JCV DNA in biopsy material and CSF from PML patients and controls with immunosuppressive underlying diseases.

MATERIALS AND METHODS

Patients Studied

Brain tissue of 13 patients (cases P/B) with suspected PML (Table I) was obtained by biopsy and diagnosis of CNS disease was made by routine histopathological evaluation. As controls autoptic tissue of five patients with Huntington's disease was kindly provided by the MRC Brain Tissue Bank, Department of Neurological Surgery and Neurology, Addenbrook's Hospital, Cambridge, UK. Samples were taken from caudate nucleus or putamen that was severely affected by the disease. In 12 cases with clinical symptoms consistent with PML (cases P/C; Table I) CSF was evaluated by PCR. PML was diagnosed on the basis of clinical course and neuroimaging features (seven cases) or by routine histopathological examination either of biopsy or of autopsy material (five cases). CSF was additionally collected by lumbar puncture from HIV seropositive patients without CNS symptoms (12 cases) and from 11 individuals with multiple sclerosis. Fresh biopsy material and CSF were extracted by lysis with SDS (final concentration 1%) followed by deproteinization under slight movement at 37°C and phenol/chloroform extraction [Dörries, 1984]. Finally, DNA was concentrated by ethanol precipitation and stored at 4°C in 10 mM Tris/HCL, 1 mM EDTA, pH 7.4.

Amplification of Virus-Specific DNA by Polymerase Chain Reaction (PCR)

For PCR, viral DNA from each case was amplified with the early region primer pair 57 and PEP-2 [Dörries et al., 1994] at 5 pmol in a reaction volume of 50 μl with 1U taq polymerase (Gibco-BRL, Gaithersburg, MD) in the recommended buffer supplemented with 2.5 mM $MgCl_2$ and 0.1% NP40. Amplification of viral DNA was achieved by 40 cycles of 1.5 min at 52°C, 4 min at 72°C, and 1.5 min at 94°C following an initial denaturation step of 5 min at 94°C. Reactions were performed in parallel with negative and positive controls in a laboratory exclusively used for PCR. All reagents were pretested for the presence of polyomavirus specific DNA. Electrophoretic separation of the products was in 4% Nusieve/GTG agarose (3:1; FMC Bioproducts, Rockland, ME) in Tris-acetate buffer at 10 Volt/cm. Standard alkaline blotting was performed overnight onto nylon membranes. The specificity of the products was analyzed by radioactive hybridization with a JCV specific internal oligonucleotide probe (JEP-1) after labeling with T4 polynucleotide kinase (γ^{32}P-ATP, spec. act. 3,000 Ci/mMol) to a specific activity of about 5×10^7 cpm/μg [Arthur et al., 1989]. The annealing reaction was carried out overnight at 55°C in a buffer of 6 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 250 μg salmon sperm DNA/ml at 10^7 cpm/cm^2. Washing conditions were in 6 × SSPE, 1% SDS at room temperature and 10 min at 70°C. PCR of the viral control region with flanking late coding DNA sequences was performed with 5 pmol of each primer (JC)71/72 [Dörries et al., 1994]. Buffer and cycling were as described above with annealing conditions of 60°C. For the second reaction an internal primer pair (JC)53 (-GCC TCC TAA AAA GCG TCG ACG CCC TTA CTA C-) and (JC)61 (-CTG TCT TCA CCT GTG CAA AAG TCC AGC-) with an annealing temperature of 65°C was used. All primers were selected by the computer program 'Primer Analysis Software Oligo 4.0' (National Biosciences, Plymouth, MN). For characterization of the products electrophoretic separation and blotting was performed as described above or in 1% agarose gels followed by standard blotting procedures. Hybridization was done with radioactively labeled JCV specific ssRNA probes as described earlier [Dörries et al., 1994]. Exposures were made with DuPont Cronex Lightning Plus enhancer screens on Cronex 2 films. The presence of inhibitors in CSF samples was investigated by addition of cloned virus DNA to CSF samples in the femtogram range. Specimens were considered inhibitor free if the concentration of amplification products was similar to that of control CSF samples with the same amount of cloned virus DNA.

Molecular sensitivity of the PCR system was comparable in concentrations from 5 ng to 0.5 fg if DNA of the two JCV subtypes GS/B and Mad-1 was used [Frisque et al., 1984; Loeber and Dörries, 1988]. Specificity of the PCR products was additionally analyzed in selected cases by nucleotide sequencing of the conserved early part of the JCV control region. Products generated by the degenerative primer pair 71/72 carried cloning sites EcoRI and BamHI, respectively [Dörries et al., 1994]. After cloning in the vector pBluescript KS(+) double-strand-dideoxy-sequencing was performed with vector or virus DNA specific primers and sequenase (USB/Amersham, Scotland) [Dörries et al., 1994].

TABLE I. Underlying Disorder and Method Leading to Diagnosis of PML

Case	Diagnostic method	Underlying disease
P/B 1[a]	clin/radiol[b]	AIDS
P/B 5	clin/radiol	none
P/B 7	clin/radiol	none
P/B 10	clin/radiol	non-Hodgkin's lymphoma
P/B 11	clin/radiol	none
P/B 12	clin/radiol/autopsy	none
P/B 13	clin/radiol	sarcoidosis
P/B 21	clin/radiol	systemic lupus erythematosus
P/B 24	clin/radiol/autopsy	tuberculosis
P/B 28	clin/radiol/autopsy	lymphoma
P/B 29	clin/radiol	not available
P/B 30	clin/radiol	AIDS
P/B 37	clin/radiol	AIDS
P/C 1[c]	clin/radiol	Hodgkin's disease
P/C 2	clin/radiol	diabetes
P/C 3	clin/radiol/biopsy	not available
P/C 4	clin/radiol/biopsy	systemic lupus erythematosus
P/C 5	clin/radiol/biopsy	AIDS
P/C 6	clin/radiol/autopsy	AIDS
P/C 7	clin/radiol/autopsy	AIDS
P/C 8	clin/radiol	AIDS
P/C 9	clin/radiol	AIDS
P/C 10	clin/radiol	AIDS
P/C 11	clin/radiol	AIDS
P/C 12	clin/radiol	AIDS

[a]Group of patients with suspected PML at time of biopsy.
[b]Diagnosis after clinical and radiological criteria [Berger et al., 1987; Krupp et al., 1985; vonEinsiedel et al., 1993].
[c]Group of patients analyzed by JCV specific PCR of cerebrospinal fluid.

TABLE II. Diagnosis After Detection of JCV DNA by Direct Southern Blotting or PCR of Cellular DNA From Brain Biopsies

Cases	Type of biopsy	Presence of JCV as detected by: Southern blot[a]	PCR[b]	Diagnosis[c]
P/B 10	open	+	+	PML
P/B 11	open	+	+	PML
P/B 12	open	+	+	PML
P/B 28	open	+	+	PML
P/B 29	open	+	+	PML
P/B 24	open	+	+	non-PML
P/B 7	open	−	−	non-PML
P/B 30	open	nd	−	non-PML
P/B 1	stereotactic	nd	+	PML
P/B 13	stereotactic	nd	+	PML
P/B 21	stereotactic	nd	−	PML
P/B 37	stereotactic	nd	−	PML
P/B 5	stereotactic	nd	−	non-PML

[a]Detected by Southern blot analysis and radioactive JCV specific hybridization.
[b]Characterization of PCR amplicons by radioactive hybridization of Southern blots with JCV specific RNA (control region) or oligonucleotides (early coding segments).
[c]PML diagnosis after histopathologic evaluation, Southern blot, and/or PCR of tissue.

RESULTS

Diagnosis of PML by Detection of JCV DNA in Biopsy Material

Brain tissue of patients suspected of having PML by neurological symptoms and clinical course was obtained by open (eight) or stereotactic (five) procedures for routine diagnosis. Basic disorders included malignant diseases, sarcoidosis, lupus erythematosus, tuberculosis, and AIDS. Four of the patients had no detectable impairment of immune function (Table I). JCV specific DNA sequences were detected in biopsy material by Southern blot analysis and/or by PCR followed by hybridization with radioactive virus specific probes. Amplification of JCV DNA was achieved either by a primer pair spanning a 199 bp fragment of the early coding DNA segment or primers spanning the complete noncoding control region and late coding sequences. The resulting product had a variable length of about 600 bp according to the JCV subtype present in the tissue [Frisque et al., 1984; Loeber and Dörries, 1988].

DNA extracts from open biopsies of eight patients with PML symptoms were analyzed by Southern blot hybridizations and PCR amplification of 1 μg cellular DNA (Table II). Five of the specimens ($P/B_{10,11,12,28,29}$) were found to be positive with clear JCV specific autoradiographic signals, suggesting PML. Two specimens in this group were negative for JCV DNA ($P/B_{7,30}$) pointing to another cause of disease. In these cases histopathological examination of additional biopsy samples or of tissue taken by autopsy was in line with the virological diagnosis. One other case (P/B_{24}) revealed faint autoradiographic bands specific for JCV DNA in Southern blot analysis. Because of the low amount of virus specific DNA, and atypical histological changes, Southern blot analysis and additional histopathological examination remained questionable. PCR on the same specimen showed amplification of JCV target DNA, and based on the PCR result PML was assumed. At autopsy the diagnosis of PML could not be confirmed. This was essentially due to the lack of the typical PML associated cell pattern and cytolytically infected oligodendrocytes. The presence of JCV DNA was therefore interpreted as an activated persistent infection suggesting that the detection of JCV DNA by the highly sensitive PCR system alone is not enough for a safe diagnosis.

In stereotactic biopsy (five cases), the amount of individual samples allowed diagnosis by PCR only. In two (of five) patients ($P/B_{1,13}$) JCV DNA was amplified, and PML was confirmed by histopathology. In the other three cases ($P/B_{5,21,37}$) amplification remained negative. In case P/B_{37} the clinical/radiological diagnosis of PML was confirmed by autopsy. In case P/B_{21} another of the serial biopsy samples was found positive for PML in histopathological examination. Thus, two of the PML cases, that underwent stereotactic biopsy were diagnosed as PML negative by PCR, whereas P/B_5 was a true negative belonging to the group of non-PML patients with CNS lymphoma. As a control group with other neurological diseases autoptic tissue from five immunocompetent patients with Huntington's disease was analyzed for the presence of JCV DNA. In each case one sample was taken from caudate nucleus or putamen, regions of active disease, and analyzed by

Southern blot and diagnostic PCR. In none of these specimens was JCV DNA detected.

Thus, in seven out of eight cases (87.5%) molecular diagnosis on open biopsies was similar to that of histopathological evaluation; only one case was falsely diagnosed as PML after PCR analysis. In five stereotactic biopsies no false diagnosis of PML was generated, although two PML cases were not recognized. In contrast, in normal, immunocompetent patients with nonrelated CNS disease all samples were found negative. This finding demonstrated that persistent JCV infection in the central nervous system in isolated cases of the high risk groups might interfere with PCR dependent diagnosis of PML.

Presence of JCV DNA in CSF of PML Patients

In the attempt to demonstrate JCV that most likely was shed into the ventricular spaces in PML patients, DNA was extracted from cell free CSF and than subjected to Southern blot analysis after cleavage with the restriction enzyme BamHI rendering linearized genomic DNA. Since the method of direct molecular detection followed by radioactive hybridization with a JCV specific radioactive probe does not include an amplification step, volumes of 2–5 ml CSF were analyzed per case. Under these conditions from 10 PML patients only one revealed the presence of faint JCV specific DNA bands of 5 kb in length representing full length JCV DNA. Thus the detection method was far too insensitive to serve as a diagnostic procedure.

The JCV specific PCR detection systems appeared to solve the dilemma. Amplification of a fragment in the early coding region and the noncoding region of virus specific DNA in CSF achieved a detection limit of about 0.5 femtogram cloned pJCV DNA or of about 100 genome equivalents as evaluated by reconstruction experiments. PCR analysis was performed on CSF of seven cases with clinically and radiologically diagnosed PML ($P/C_{1,2,8-12}$) and of five cases with histopathologically confirmed PML (P/C_{3-7}) (Table III). Initial experiments with a CSF volume equivalent of 50 μl revealed amplification of virus specific products in three PML patients only (3/12 cases, 25%). Therefore an equivalent of 250 μl was analyzed in a reaction with the early region primer pair. Under those conditions five of 12 patients (41.7%) exhibited a positive result after electrophoretic separation of the PCR products. Southern blot of the products and hybridization with a JCV specific radioactive oligonucleotide revealed the presence of JCV specific DNA segments in a sixth case (6/12 cases, 50%). This pointed to a detection limit too low in some of the cases (Table III).

To enhance the sensitivity of the PCR system a CSF equivalent of 500 μl was analyzed. In this reaction JCV products were amplified in three additional CSF samples after electrophoretic separation with no further enhancement by radioactive Southern blot analysis (5/9 cases, 56%). An increase of the CSF volume to 1,000 μl in the case of negative specimens ($P/C_{3,4}$) did not improve the detection rate. Reaction with the control region primer pair spanning the noncoding region and additional late coding sequences revealed similar results. Inhibition tests with the CSF of negative cases clearly demonstrated that no inhibitory factors were present in the CSF that might interfere with the enzyme reaction.

TABLE III. Presence of JCV Sequences in CSF of PML Patients

	CSF volume equivalents analyzed in PCR[a,b] and JCV specific characterization of products[c]				
Cases	Minimal CSF equivalent found positive (μl)	1. PCR 250	500	1000	PCR nested[d]
P/C 1	50	+[b]/+[c]	+/+		+/+
P/C 2	500	−/−	+/+		+/+
P/C 3	>500	−/−	−/−		+/+
P/C 4	>500	−/−	−/−		+/+
P/C 5	50	+/+			+/+
P/C 6	—	−/−	−/−	−/−	−/−
P/C 7	—	−/−	−/−	−/−	−/−
P/C 8	250	−/+	+/+		+/+
P/C 9	50	+/+	+/+		+/+
P/C 10	100	+/+			+/+
P/C 11	500	−/−	+/+		+/+
P/C 12	125	+/+			+/+
JCV positive total		6/12 50%	5/9 56%		10/12 83%

[a]First, standard PCR with primer pairs for a JCV early coding DNA segment and the control region segment.
[b]Products characterized by gel electrophoresis.
[c]JCV specific hybridization with radioactively labeled internal oligonucleotides or virus specific RNA probes.
[d]Results of the nested PCR with internal primers on a 1 μl volume.

Aiming to enhance the detection limit further nested PCR with internal primer pairs was used. This combines improved virus specificity with higher sensitivity of the reaction by a second round of amplification on JCV primary amplicons. For the nested PCR 1 μl of the first reaction was analyzed with the nested primer pair. In case of the detection of product amplification in the first reaction ($P/C_{1-4,8-12}$) this was performed on a CSF sample in volume equivalents found negative. In all these specimens amplification was JCV specific as shown by virus specific hybridization. Although products in nested reactions cannot be quantitated, the sensitivity of detection was considerably higher in the second reaction (50–100 times; own observations), therefore nested reactions were performed on a CSF volume between 500 μl and 1000 μl of specimens found negative in the first PCR. Under those conditions JCV specific amplification could be demonstrated in two additional PML cases ($P/C_{3,4}$; Table III). However, two cases proven as PML after autopsy ($P/C_{6,7}$) remained negative even after nested PCR analysis of more than 1,000 μl of CSF. This confirmed that a CSF volume equivalent of 500 μl produced the highest specificity and sensitivity and demonstrated JCV DNA in 10 of 12 PML patients (83%) under the test conditions used.

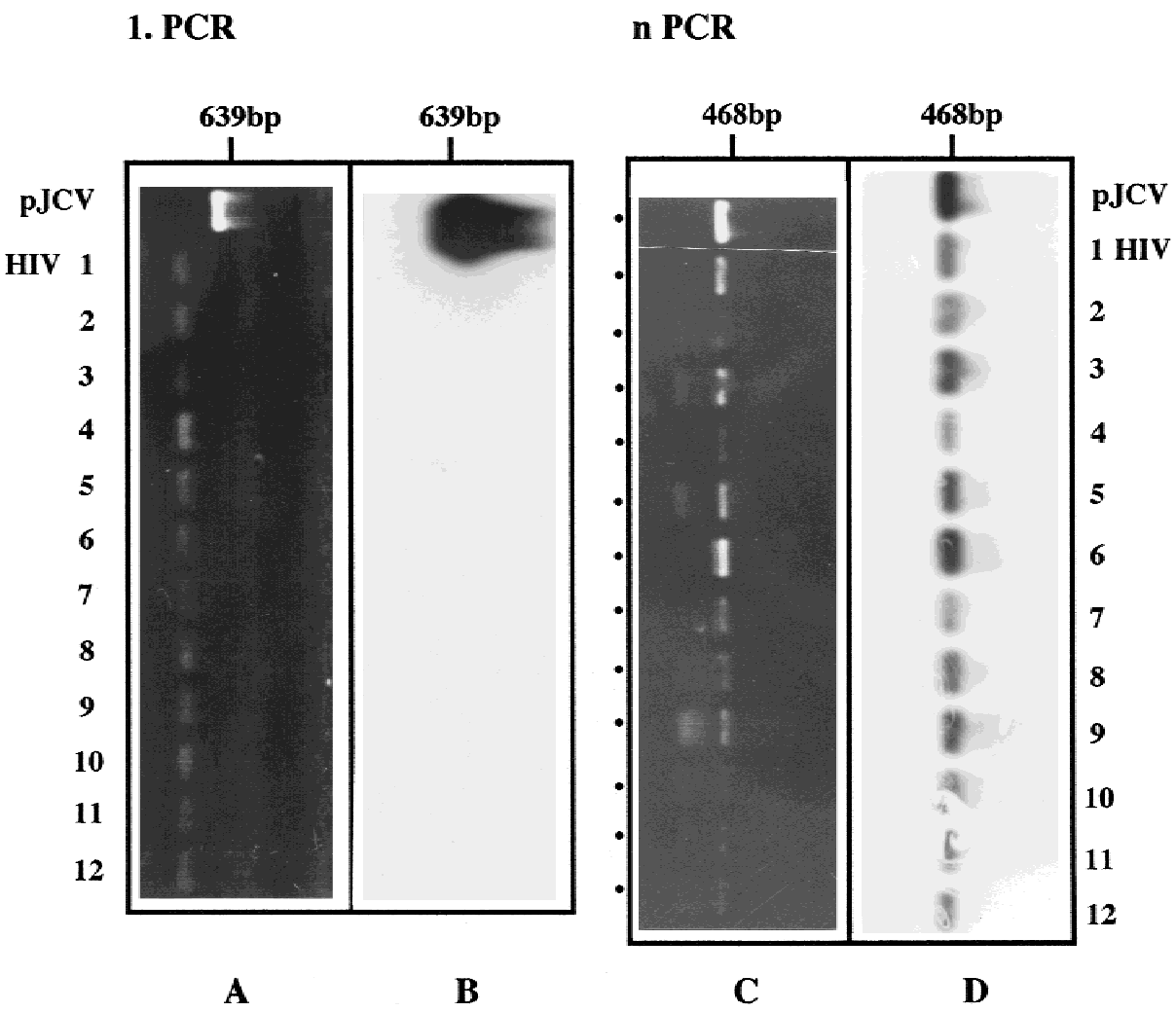

Fig. 1. Presence of JCV DNA amplicons in the CSF of AIDS patients. DNA extracts of volume equivalents of 500 μl cell free CSF were subjected to PCR with JCV control region primers. Products were analyzed by agarose gel electrophoresis and Southern blot analysis with a radioactive JCV specific probe. From the first reaction products 1 μl was removed and subjected to a second reaction with a nested primer pair. Analysis of the products was performed similarly. **A:** First PCR reaction (1.PCR). **B:** Southern blot and autoradiography of the first reaction products. **C:** Nested PCR reaction (nPCR). **D:** Southern blot and autoradiography of the nested reaction products. Product length in the 1.PCR: about 600 bp, in the nPCR: about 400bp depending on the JCV subtype present. pJCV: GS/K DNA (50 ng) control. HIV1-12: CSF specimens from individual cases; dots represent the respective specimens in C.

JCV PCR of CSF From HIV-1 Infected Patients

In previous reports, analyses of multiple CNS tissue specimens from non-PML patients and healthy individuals had shown that JCV DNA was occasionally present in the brain. The conclusion was drawn that JCV reached the CNS in the course of persistence, and might there be disseminated by repeated activation events without manifestation of disease [Elsner and Dörries, 1992; Mori et al., 1992; Quinlivan et al., 1992; White et al., 1992; Dörries, 1997]. This implicated the possibility of false positive diagnosis after PCR on the CSF due to putative JCV activation unrelated to PML. Therefore we analyzed CSF from patients of the high risk HIV-1 seropositive group without neurological symptoms in parallel with a group of multiple sclerosis patients (11 cases) not at risk for PML. CSF from 12 HIV-1 patients were subjected to JCV PCR. All specimens were analyzed with early coding and control region primers at a CSF equivalent of 500 μl (Table IV). The products were characterized under identical conditions as in PML cases. In none of the cases (0/12) PCR products could be visualized after electrophoretic separation and radioactive Southern blot analysis (Fig. 1A,B) in the first reaction. However, nested PCR analysis demonstrated the presence of JCV specific products in 12 of 12 specimens after electrophoretic separation of the PCR products and radioactive virus specific hybridization. The concentration of amplicons appeared to be variable according to product bands after electrophoretic separation. The result was confirmed in several consecutive experiments. Specificity of the amplified DNA segment was additionally confirmed by DNA

TABLE IV. Examination of Polyomavirus JC Sequences in CSF of Non-PML Patients With AIDS

Basic disease	Number of patients	$CD4^+/CD8^+$ ratio	PCR product[c]			
			1.[a]	JCV[c]	n[b]	JCV[c]
AIDS	1	nd[d]	0/1	0/1	1/1	1/1
	3	0.02–0.03	0/3	0/3	3/3	3/3
	4	0.15–0.27	0/4	0/4	4/4	4/4
	4	0.66–1.35	0/4	0/4	4/4	4/4
total	12		0/12	0/12	12/12	12/12

[a]1. JCV PCR reaction with 500 µl CSF volume equivalent, primers used for the early coding and control region DNA segments.
[b]Nested PCR reaction with the internal control region primer pair.
[c]JCV specific hybridization.
[d]nd, Not done.

sequencing from selected patients. In all cases the DNA sequence was corresponding to the conserved early part of the JCV control region. In contrast to the HIV infected cases, CSF samples from the immunocompetent group of multiple sclerosis patients remained negative for JCV DNA in conventional and nested PCR analyses.

Assuming that the state of the immune system might be responsible for the presence of JCV in the CSF, patients were grouped according to their ratios of $CD4^+/CD8^+$ T lymphocytes at the time of CSF sampling (Table IV). PCR analysis revealed similar results in all groups and suggested that presence of JCV DNA in the CSF was not dependent on changes in the ratio of $CD4^+/CD8^+$ T lymphocytes in this group of patients. Therefore this parameter was not helpful for the differential diagnosis of apathogenic JCV infection and PML.

DISCUSSION

Virological diagnosis of polyomavirus associated diseases has not yet reached routine standards. PML cases often are diagnosed retrospectively, although the importance of polyomaviruses as opportunistic agents in AIDS is reflected in a steadily increasing number of cases [Major and Ault, 1995]. Accumulating knowledge on the viral life cycle and use of the new sensitive detection systems additionally complicates discussion of diagnostic results [Dörries, 1997]. Thus, for future development of a standardized regimen, it is essential to evaluate technical approaches in view of an activated subclinical state of JCV infection in the CNS.

Prior to the utilization of PCR, diagnosis of JCV CNS infection was made by identification of typical cellular changes and virus products in biopsy material. The extraordinary multiplication rate of the virus in diseased tissue [Dörries et al., 1979, Walker and Padgett, 1983] allowed diagnosis of PML by classical techniques with a high sensitivity and specificity [Kepes et al., 1975; Gibson et al., 1986; Aksamit et al., 1987; Gosztonyi and Cervos-Navarro, 1988; Gardner and Knowles, 1994]. The introduction of PCR, however, was followed by diagnoses of PML that could not be confirmed after autopsy [Gibson et al., 1986]. The discrepancy of the results is probably due to the differential amount of JCV DNA in PML tissue and in the rare patient with an activated persistent JCV infection. After intensive analytical search by high sensitivity PCR [Brun et al., 1973; Kepes et al., 1975; Elsner and Dörries, 1992; Quinlivan et al., 1992; White et al., 1992; Berger and Concha, 1995; Ferrante et al., 1995] it is now widely accepted that polyomaviruses asymptomatically persist in the CNS. This state of infection is regularly not detected by diagnostic conventional PCR analysis of single brain samples. However, the detection of JCV DNA in a non-PML brain by diagnostic PCR demonstrates that differences of the amount of JCV DNA in PML associated with cytolytic infection, and in a subclinically activated persistent state cannot not be detected by conventional PCR. It can be assumed that quantification of JCV DNA in biopsy specimens will help to differentiate a persistent and the PML associated JCV infection. At present, we evaluate quantitative PCR for PML diagnosis.

The failure to detect JCV specific DNA in single samples of serial stereotactic biopsy specimens [Moret et al., 1993] was most likely due to sampling conditions. The characteristic morphology of PML lesions with an outer rim of active virus growth surrounding an almost virus-free center might represent a higher risk of removing material from non-affected tissue in stereotactic biopsy than in open biopsy. Since open surgery for PML diagnosis is almost completely replaced by stereotactic biopsy, precise topographical selection of samples is necessary for correct molecular virological diagnosis. Thus, at present, a combination of stereotactic biopsy, PCR and direct in gel characterization of amplicons ensure a fast, sensitive and specific diagnosis of PML.

The problems associated with brain surgery determine the need for less invasive methods in the diagnosis of PML [Karahalios et al., 1992]. In recent years, several laboratories have shown that JCV target DNA in CSF of PML patients can be detected by PCR at high sensitivities [Gibson et al., 1993; Moret et al., 1993; Weber et al., 1994; Fong et al., 1995; DeLuca et al., 1996]. However, the reports documented divergent JCV DNA detection rates ranging from 17 to 100%. In addition, diagnosis of PML appeared to be less specific than previously described [Cinque et al., 1993; Fong et al., 1995; DeLuca et al., 1996]. Differences in the amplification techniques were discussed as an explanation for the discrepancies between laboratories. The most prominent factor was the quality of PCR primers [Weber et al., 1994]. Computer programs for the selection of primer pairs improved the test systems resulting in comparable detection limits, at present ranging from 1 to 0.1 fg JCV DNA (200 to 20 JCV genomes) [Gibson et al., 1993; Bogdanovic et al., 1994; Fong et al., 1995]. Additionally, sample preparation and CSF volumes reported for PCR are highly variable. Either standard DNA extraction methods are applied prior to PCR [Gibson et al., 1993; Weber et al., 1994; Fong et al., 1995] or CSF is directly used after boiling [Moret et al., 1993;

Fong et al., 1995; DeLuca et al., 1996]. Although the extraction technique might be associated with a loss of viral DNA per volume equivalent, considerably higher amounts of CSF can be analyzed in one reaction and the influence of inhibitory factors on the performance of the PCR is reduced [Gibson et al., 1993; DeLuca et al., 1996].

In most reports CSF volumes between 1 and 100 μl CSF were subjected to PCR [Telenti et al., 1990; Henson et al., 1991; Bogdanovic et al., 1994; Weber et al., 1994; Fong et al., 1995; DeLuca et al., 1996]. Detection rates for JCV ranging between 17% and 100% in CSF of PML patients suggest that the volume of CSF used in PCR might be essential for a fast and reliable diagnosis. In the attempt to analyze the influence of the CSF volume on the diagnosis by PCR we used different amounts of CSF and depending on the CSF volume the detection rate could be enhanced from 25% to 56% of the PML cases. The detection limit of the applied test system for JCV was in the range of less than 200 genome equivalents per reaction and therefore comparable to that described by DeLuca et al. [1996], Moret et al. [1993], and Fong et al. [1995], but lower than that of Gibson et al. [1993] and Weber et al. [1994]. Higher sensitivities were achieved after applying nested PCR reactions. In this case JCV DNA was demonstrated in 83% of the PML patients. This is in the best range of detection rates reported and similar to that observed in patients after biopsy. However, in our study and other reports PML cases remaining negative even after nested PCR point to additional [Gibson et al., 1993; DeLuca et al., 1996] factors, that might be essential for PML PCR diagnosis on CSF. Since control reactions clearly demonstrated the absence of inhibitory factors in these PML cases an interpretation of the result cannot be given, and the question of why those patients might evade PCR diagnosis remains open.

In view of a subclinical persistent JCV infection in the CNS and possible activation processes, that might pass unnoticed, PCR was performed on the CSF of the high risk HIV patients without neurological symptoms at different states of immunosuppression. In all those cases the standard PCR was negative up to a CSF equivalent of 2 ml. In nested reactions, however, JCV DNA fragments were amplified from all patients. This is in line with findings of JCV DNA in CSF of HIV infected patients without PML in three other laboratories [Cinque et al., 1993; Fong et al., 1995; DeLuca et al., 1996] clearly demonstrating that JCV can be detected by nested PCR on CSF of the high risk groups. Thus the nested reaction is a tool too sensitive to differentiate a subclinical persistent JCV infection and the cytolytic state under PML. Consequently, diagnosis of PML is limited to the standard PCR technique and the associated sensitivity of 60%–70%. In contrast to HIV patients, in the CSF of immunocompetent patients with MS in none of the samples analyzed by PCR JCV DNA was amplified. This confirms earlier findings on a higher concentration of JCV DNA in immunosuppressed individuals [Elsner and Dörries, 1992] and suggests that subclinical activation of a persistent JCV infection in the CNS might be limited to the high risk groups of patients [Brooks and Walker, 1984].

Of the diagnostic procedures at present available for PML, brain biopsy is that of the highest sensitivity and specificity. Combined with quantitative analyses serial stereotactic sampling probably allows unequivocal results. In contrast, the presence of JCV DNA in the CSF of asymptomatic HIV patients and absence of JCV in PML patients demonstrates that diagnosis of PML cannot be achieved by PCR analysis on CSF alone. Even repeated CSF sampling might not lead to a safe diagnosis as long as the kinetic of the JCV infection in high risk patients and the amount of virus DNA present in different states of infection is not examined in a higher number of patients. Therefore, until further elucidation of the mechanisms leading to JCV activation and disease, PCR must be understood as a diagnostic tool used in combination with clinical and radiological findings. Conditions for a diagnostic regimen leading to PML diagnosis by combination of clinical, radiological, and CSF diagnosis were summarized recently [vonGiesen et al., 1997]. Although a therapy for PML is not yet established, in cases where PML is the only AIDS defining opportunistic disease, in patients under immunosuppressive therapy, and as a differential diagnosis in cases of CNS lymphoma unequivocal diagnosis might be necessary that can only be established by biopsy.

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